The World Journal of Biological Psychiatry, 2014; 15: 334–345

ORIGINAL INVESTIGATION

The Tutsi genocide and transgenerational transmission of maternal stress: epigenetics and biology of the HPA axis

NADER PERROUD[1,2], EUGENE RUTEMBESA[3], ARIANE PAOLONI-GIACOBINO[4], JEAN MUTABARUKA[3], LÉON MUTESA[5], LUDWIG STENZ[2], ALAIN MALAFOSSE[2,4,*] & FÉLICIEN KAREGE[4,6*]

[1]*University Hospitals of Geneva, Department of Mental Health and Psychiatry, Geneva, Switzerland,* [2]*University of Geneva, Department of Psychiatry, Geneva, Switzerland,* [3]*University of Rwanda, Faculty of Medicine, Department of Clinical Psychology, Butare, Rwanda,* [4]*University Hospitals of Geneva, Department of Medical Genetics and Laboratory, Geneva, Switzerland,* [5]*University Teaching Hospital of Kigali, Faculty of Medicine, Department of Medical Genetics, Kigali, Rwanda, and* [6]*University of Rwanda, Faculty of Science, Department of Biology, Butare, Rwanda*

Abstract

Objectives. Transmission of parental post-traumatic stress disorder (PTSD) to offspring might be explained by transmission of epigenetic processes such as methylation status of the glucocorticoid receptor (GR) gene (*NR3C1*). *Methods.* We investigated PTSD and depression severity, plasma cortisol, GR and mineralocorticoid receptor (MR) levels, and methylation status of *NR3C1* and *NR3C2* promoter regions in 25 women exposed to the Tutsi genocide during pregnancy and their children, and 25 women from the same ethnicity, pregnant during the same period but not exposed to the genocide, and their children. *Results.* Transmission of PTSD to the offspring was associated with transmission of biological alterations of the HPA axis. Mothers exposed to the genocide as well as their children had lower cortisol and GR levels and higher MR levels than non-exposed mothers and their children. Moreover, exposed mothers and their children had higher methylation of the *NR3C1* exon 1_F than non-exposed groups. Finally, exposed mothers showed higher methylation of CpGs located within the *NR3C2* coding sequence than non-exposed mothers. *Conclusions.* PTSD was associated with *NR3C1* epigenetic modifications that were similarly found in the mothers and their offspring, modifications that may underlie the possible transmission of biological alterations of the HPA axis.

Key words: *PTSD, NR3C1, NR3C2, glucocorticoid, HPA axis*

Introduction

Between April and June 1994, almost one million people died in the Rwandan genocide against ethnic Tutsi. The long-term impact of this dramatic event was still visible in 2011 with more than 20% of the general population meeting a diagnosis of depression and/or post-traumatic stress disorder (PTSD) (Munyandamutsa et al. 2012). Not surprisingly, PTSD was highly prevalent in the years following the genocide. In 1995, a large survey of Rwandans aged between 8 and 19 years old revealed that 50–60% of them fulfilled criteria for PTSD (Neugebauer et al. 2009). In this survey, the estimated prevalence of PTSD among widows was 41.4%, prevalence confirmed by later studies in the field (Schaal et al. 2011; Munyandamutsa et al. 2012). These studies are proof of the massive and enduring impact of the genocide on the widows who either witnessed or endured these horrible events.

Several studies have suggested that parental PTSD is a strong correlate for the development of PTSD in offspring (Brand et al. 2006; Yehuda and Bierer 2009; Brand et al. 2011). This transmission is additionally associated with transmission of biological alterations of the hypothalamic–pituitary–adrenal (HPA) axis. PTSD is indeed associated with alteration

*Joint last authors.
Correspondence: Prof Félicien Karege, University of Rwanda, Faculty of Science, Department of Biology. P.O.Box 117 Buatre, Rwanda. Tel: +250 788618445. E-mail: felicien.karege@gmail.com

(Received 7 July 2013; accepted 6 November 2013)
ISSN 1562-2975 print/ISSN 1814-1412 online © 2014 Informa Healthcare
DOI: 10.3109/15622975.2013.866693

of the HPA axis mainly in the sense of low cortisol levels (Brand et al. 2006; Yehuda and Bierer 2009; Brand et al. 2011) and this low level of cortisol alongside an increased cortisol suppression in response to dexamethasone has been observed in offspring of trauma survivors with PTSD (Yehuda et al. 2007; Yehuda and Bierer 2009). This low cortisol level in relation to parental PTSD appears to be present early in the course of development, probably in utero and may be the consequence of glucocorticoid programming through epigenetic processes and thus on gene expression (Brand et al. 2006).

These epigenetic programming processes have been studied in greater detail for the gene coding for the glucocorticoid receptor (*NR3C1*). Previous studies have indeed shown that environmental stressors, not only early in life, but also during pregnancy, increase methylation status of the exon 1_F *NR3C1* promoter region in a way that is still measurable later in life (Oberlander et al. 2008; Radtke et al. 2011). This phenomenon is believed to durably perturb the HPA axis and may account for the altered cortisol levels found in offspring of trauma survivors with PTSD. Thus epigenetics may provide an important insight into the pathophysiology of PTSD since it might explain the transgenerational transmission of that disorder. In the light of this hypothesis, Mulligan et al. (Mulligan et al. 2012) recently showed that extreme maternal psychosocial stressors, as observed in the Democratic Republic of Congo, modified locus specific epigenetic marks in the newborn resulting in altered health outcomes. If the *in utero* environment appears to be an important factor related to alteration of methylation of *NR3C1* in newborns and appears to mediate neurobehavioral measures assessed at birth (Oberlander et al. 2008; Filiberto et al. 2012; Bromer et al. 2013), the impact of these altered methylation processes on later neurobehavioral outcomes remains poorly addressed.

In view of evidence of *in utero* HPA axis programming through epigenetic mechanisms and of observation of lower cortisol levels in offspring of mothers with PTSD compared to mothers without PTSD, we undertook this study to test the effect of *in utero* maternal stress, as measured by PTSD severity, on behavioral outcomes, biological correlates and methylation status of *NR3C1* promoter region in the offspring. To do so, we recruited 25 women exposed to the Tutsi genocide and pregnant at the time of the trauma and 25 women from the same ethnicity, pregnant during the same period but without being exposed to the genocide. We then assessed PTSD and depression severity, cortisol, glucocorticoid receptor (GR) and mineralocorticoid (MR) levels, and *NR3C1* and mineralocorticoid receptor *NR3C2* methylation status in these women and their children.

Materials/subjects and methods

Subjects

Twenty-five widows of Tutsi ethnicity exposed to traumatic experiences in the context of the Rwanda genocide and who were pregnant (second and third terms of pregnancy) during the genocide period, and their 25 offspring born after that period were recruited for this study between 2011 and 2012. The traumatic experience was defined according to the DSM-IV-TR criterion A: the person has experienced, witnessed, or been confronted with an event or events that involve actual or threatened death or serious injury, or a threat to the physical integrity of oneself or others; and the person's response involved intense fear, helplessness, or horror. Subjects were recruited through survivors' associations and/or psychiatric ambulatory consultations in Rwanda.

Twenty-five women from the same ethnicity who were living abroad at the time of the genocide and therefore not exposed to the traumatic experience and their 25 offspring born during the same period were recruited through different networks (high schools, corporate associations, medical contacts, etc.). A known exposure to a traumatic experience in the past as well as a history of psychiatric disorder was considered as exclusion criteria for these 25 non-exposed women.

Assessments

All subjects (mothers and children) were clinically evaluated by a trained psychologist for history of psychiatric disorders.

The PTSD 17-item checklist (PCL-17) was used to assess severity of current PTSD. The questionnaire was administered by a trained psychologist. This 17-item instrument corresponds to diagnosis B, C and D criteria for PTSD, as delineated in the DSM-IV. The PCL-17 does not enable a diagnosis of PTSD, but a total score of 44 is interpreted as warranting clinical attention (Ruggiero et al. 2006).

To assess the current severity of depression, the self-report questionnaire 13-item Beck Depression Inventory (BDI-13) was used (Collet and Cottraux 1986).

All participants gave their informed written consent and the study was approved by the Institutional Review Board of the University Teaching Hospital of Kigali (Ref EC/CHUK/025/11).

DNA extraction

DNA was extracted from peripheral blood leukocytes using the classical method of phenol/chloroform and

following manufacturer's instructions. Blood samples were stored at the Laboratory of Medical Genetics, University Laboratory of National University of Rwanda/Faculty of Medicine and DNA extracted by the same laboratory. Samples were then transferred to Geneva University Hospitals (HUG, Switzerland) within 2 months in appropriate conditions.

Plasma samples isolation

Blood collected in EDTA-containing tubes was centrifuged at 1000 $g \times 15$ min and then plasma was separated and stored at –20°C, until sent to Geneva University Hospitals (Switzerland), within 2 months in appropriate conditions.

NR3C1 methylation status

Methylation status of the exon 1_F *NR3C1* promoter region was performed at the University Hospitals of Geneva (HUG, Switzerland) as previously described (Perroud et al. 2011). Briefly, pyrosequencing was used to assess the DNA methylation status. The term "DNA methylation" will be used throughout the paper in order to be consistent with previous publications; however, due to other DNA modifications not assessed in the current paper, the more correct term should be "DNA modification". We analysed a portion of the exon 1_F *NR3C1* promoter similar to the one explored in Perroud et al. (2011) containing eight CpG sites. Two additional CpGs were added to better overlap with the one reported by McGowan and co-workers (Oberlander et al. 2008; McGowan et al. 2009). The PCR amplifications were performed starting from 100 to 140 ng of bisulfite-treated DNA. The PCR conditions were 94°C for 15 min, followed by 50 cycles of 94°C, 30 s, 52°C, 30 s, 72°C, 40 s, and by a 72°C for 10 min final extension step.

The sequence of the oligonucleotides, within *NR3C1* (GenBank # AY436590) is the following: NR3HumF1: 5′-TTTGAAGTTTTTTTAGAGG G-3′ and NR3HumR: 5′-biotin-7-CCCCCAACT CCCCAAAAA-3′ (adapted from Oberlander et al., 2008). Amplification resulted in a 403-bp fragment (position −3485 to −3082). All reactions were performed with a PCR reaction mixtures (total volume 25 μL) containing oligonucleotides at 0.5 mM concentration and 12.5 μL of HotStarTaq Master Mix (Qiagen, CA, USA). The biotinylated PCR products were purified using streptavidin-sepharose beads (Amersham) and sequenced using the PSQ 96 Gold reagent kit (Biotage AB, Uppsala, Sweden) with the following primer: NR3HumS1: 5′-GAGTGGGTT TGGAGT-3′.

The degree of methylation at each CpG site was determined using Pyro Q-CpG Software (Biotage AB, Uppsala, Sweden). Ten CpG sites were analysed and percentage of total methylation was calculated. All samples were analysed in duplicate and mean percentage was then calculated and used for the current analyses. Samples were processed and analysed blindly with respect to demographic variables, psychiatric diagnoses and childhood maltreatment history.

NR3C2 methylation status

Cytosine conversion to uracil was performed by treating 1 μg DNA for each sample with a bisulfite kit (EpiTect Bisulfite Kit, Qiagen, Germany) and according to the manufacturer's protocol. Assay design for *NR3C2* promoter methylation level was performed with MethPrimer software (Li LC, Dahiya R. 2002. MethPrimer: designing primers for methylation PCRs. Bioinformatics 18:1427-31) resulting to primers "nr3c2_F_5042013": 5′-GGGAG**TT** AA**T**TT**T**AGG**T**TG**T**T**T**AGAG-3′ (bold are C converted to T), and "nr3c2_R_5042013_bio": 5′biotine-A**A**CCACTTCTCCCT**A**CAAA**A** T**A**CAC-3′ (bold are G converted to A). PCR reactions were performed in 25-μL final volume containing 50 ng bisulfite-treated DNA, 1 × PCR buffer, 1.6 mM $MgCl_2$, 200 μM dNTP, 0.2 μM nr3c2_F_5042013 primer, 0.2 μM nr3c2_R_5042013_bio primer, 2 units of Hot Start Taq DNA polymerase (HotStart Taq DNA Polymerase Kit, Qiagen). PCR condition was as follows: 95°C for 15 min followed by 50 cycles of 95°C for 30 s, 58.5°C (determined experimentally) for 30 s, 72°C for 10 s. The biotinylated PCR products were purified using streptavidin-sepharose beads (Amersham) using a PyroMark Q96 Vacuum Prep Workstation (Qiagen) and sequenced using the PSQ 96 Gold reagent kit (Biotage AB, Uppsala, Sweden) on a Pyromark 96MA with the primer nr3c2_F_5042013 used as the sequencing primer. The degree of methylation at each CpG site was determined by using duplicate samples and with the Pyro Q-CpG Software (Biotage AB, Uppsala, Sweden).

The 300-bp DNA sequence of the assay (hg19/chr4:149-363-223-149 363 522) is located within the overlaps between a CpG island and the start of *NR3C2* gene: **G**GGAGCCAACTTCAGGCTGCT CAGAGGAAGCC**CG**$_1$TGCAGTCAGTCACCT GGGTGCAAGAG**CG**$_2$TTGCTGCCT**CG**$_3$ GGCTCTCC**CG**$_4$CTGCAGGGAGAG**CG**$_5$ GCACT**CG**$_6$CTGGCCTGGATGTGGTT GGATTTAGGGGGGCTC**CG**$_7$CAGCA GGGGTTT**CG**$_8$TGG**CG**$_9$GTGGCAAG**CG**$_{10}$ CTGCAACAGGTAGA**CG**$_{11}$G**CG**$_{12}$AG

AGA**CG**$_{13}$GACCC**CG**$_{14}$GC**CG**$_{15}$AGGCAGGTGTGTAGGGG**CG**$_{16}$**CG**$_{17}$**CG**$_{18}$G**CG**$_{19}$GGGCAC**CG**$_{20}$CTTGC**CG**$_{21}$TGCT**CG**$_{22}$G**CG**$_{23}$TG**CG**$_{24}$GC**CG**$_{25}$**CG**$_{26}$G**CG**$_{27}$**CG**$_{28}$GGAG**CG**$_{29}$TGCACTTTGCAGGGAGAAGTGGCT. On the 29 CpG sites analyzed by pyrosequencing for methylation percentages of *NR3C2* only the eight first ones located in the promoter region of the gene were available for the analyses. Indeed, due to technical issues (decreasing signal intensity moving away from the primer binding site), for the 21 remaining CpGs we observed a continuous decrease in the number of subjects for which the methylation status was available reaching almost zero subjects for the CpG29. These CpGs were thus drop-out from the primary analyses. Thus, only the first eight CpG sites (for which all subjects were available) were analyzed and percentage of total methylation was calculated at first stage. Secondarily, due to the limited number of subjects, only comparisons between exposed and non-exposed mothers and between exposed and non-exposed children were made for CpG 9 to 29.

Protein assays: cortisol, mineralocorticoid and glucocorticoid plasma levels

Plasma cortisol. The plasma was collected for a complementary test of cortisol. Since there is a physiological fluctuation of human cortisol, achieving the highest level in the morning and the lowest at night, all blood samples were collected in the morning (between 8 a.m. and 10 a.m.) into EDTA-containing tubes. Plasma was then isolated by centrifugation (1000 $g \times 15$ min at 4°C), and stored at –20°C, before transfer to Geneva University Hospitals, for analysis.

Cortisol levels were measured with an ELISA kit based on the competition principle, according to the vendor's instructions (IBL International, Hamburg, Germany). An unknown amount of antigen present in the sample and a fixed amount of enzyme labelled antigen compete for the binding sites of the antibodies coated onto the wells. After incubation, the wells are washed to stop the competition reaction. After the substrate reaction, the intensity of the developed colour is inversely proportional to the amount of the antigen in the sample. Results of samples can be determined directly using the standard curve. Cortisol levels are expressed in ng/ml in the following results.

Glucocorticoid (GR) and mineralocorticoid (MR) levels. MR and GR plasma proteins were assayed with an ELISA method, which is a quantitative sandwich immunoassay technique, according to the supplier's instructions (MyBiosource Inc., San Diego, CA, USA). According to the supplier, the kits are based on rabbit anti-human polyclonal antibodies. In brief, antibody specific either for *NR3C1* or *NR3C2* was pre-coated onto a microplate. Samples and standards were pipetted into the wells and any *NR3C1* or *NR3C2* present in the samples was bound. After removing the unbound substances, a biotin-conjugated antibody specific for *NR3C1* or *NR3C2* was added to the wells. After washing, avidin-conjugated HRP was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of the protein (MR or GR) bound in the initial step. The colour development was stopped and the intensity of the colour was measured using plate reader at 450 nm. The method is sensitive (typically less that 1.56 pg/ml), and specific for MR and GR (no cross-reactivity with analogues, according to the supplier). The inter-assay and intra-assay variation is CV $< 8\%$ and CV $< 1\%$, respectively. MR and GR levels are expressed in ng/ml in the following results.

Statistics

To assess potential association between the four groups (exposed and non-exposed mothers and children) and the biological variables (cortisol levels, GR and MR levels), chi-square tests and *t*-tests were used. Linear regression with standardized values of methylation for each CpG and total mean of CpGs was used to assess association between exposed and non-exposed subjects and *NR3C1*/*NR3C2* methylation status. Standardized values were used as they may be interpreted as effect sizes. Significance was set at $P < 0.05$. Statistical analyses were carried out using STATISTICA, version 7 and SPSS version 18.0.

Results

The mean age of the women at childbirth was 28 ± 6 years (32 ± 5 for exposed women and 26 ± 6 years for non-exposed women). The group of children, aged 17–18 years was composed of 29 females and 21 males (12 (48%) males in the children of the non-exposed mothers and 9 (36%) males in the group of exposed mothers). There was no significant difference in the gender ratio between exposed and non-exposed groups ($P = 0.390$).

Clinical outcomes

Mothers exposed to the genocide event had significantly higher PTSD and depression severity than

Table I. PTSD and BDI severity in control vs. exposed mothers and in control vs. exposed children.

	Mothers				Children					
	Controls		Exposed		Controls		Exposed			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	M_{exp} vs. M_{nonexp} *t;P*	Ch_{exp} vs. Ch_{nonexp} *t;P*
PTSD	37.4	16.9	57	13.6	29.4	11.8	44.5	13.4	4.52; $P<0.0001$	4.14; $P=0.0001$
BDI	9.1	8	14.9	8	7.5	8.3	16.2	10.7	2.58; $P=0.01$	3.14; $P=0.003$

non-exposed mothers (Table I). Interestingly, children born from mothers exposed to the traumatic experience showed higher PTSD and depression severity than children born from non-exposed mothers.

There was a significant correlation between the severity of PTSD and depression in mothers and the severity of PTSD and depression in their children, respectively (Pearson $r=0.367$; $P=0.01$ and Pearson's $r=0.312$; $P=0.03$, respectively) (Figure 1); the correlation being stronger in the non-exposed group for both outcomes probably due to a ceiling effect in the exposed group.

In children, there was no sex-specific effect either in PTSD scores (38.71 (SD = 15.45) vs. 33.47 (SD = 13.45); $\beta=5.24$; $P=0.236$ in females and males, respectively) or in depressive scores (12.11 (SD = 9.91) vs. 10.05 (SD = 10.58); $\beta=2.05$; $P=0.501$ in females and males, respectively).

NR3C1 methylation status

Mothers exposed to the genocide revealed higher methylation status of the *NR3C1* exon 1_F promoter region than non-exposed mothers (6.67 (SD = 2.9) vs. 5.03 (SD = 3.2); $\beta=0.44$; $P=0.07$). This was significant for CpG1 (9.18 (SD = 5.5) vs. 6.00 (SD = 3.3); $\beta=0.66$; $P=0.019$), CpG5 (10.68 (SD = 5.7) vs. 7.56 (SD = 5.1); $\beta=0.47$; $P=0.05$), and CpG8 (10.13 (SD = 6.9) vs. 6.00 (SD = 6.39); $\beta=0.57$; $P=0.039$) (Figure 2). Children born from mothers exposed to the traumatic experience had higher methylation status of the *NR3C1* exon 1_F promoter region than children born from non-exposed mothers (9.77 (SD = 3.2) vs. 5.33 (SD = 3.6); $\beta=1.18$; $P=0.0001$). This was significant for CpG3 (3.61 (SD = 3.4) vs. 1.52 (SD = 2.0); $\beta=0.70$; $P=0.012$), CpG4 (14.22 (SD = 5.6) vs. 5.56 (SD = 5.5); $\beta=1.30$; $P=2.58*10^{-6}$), CpG5 (14.17

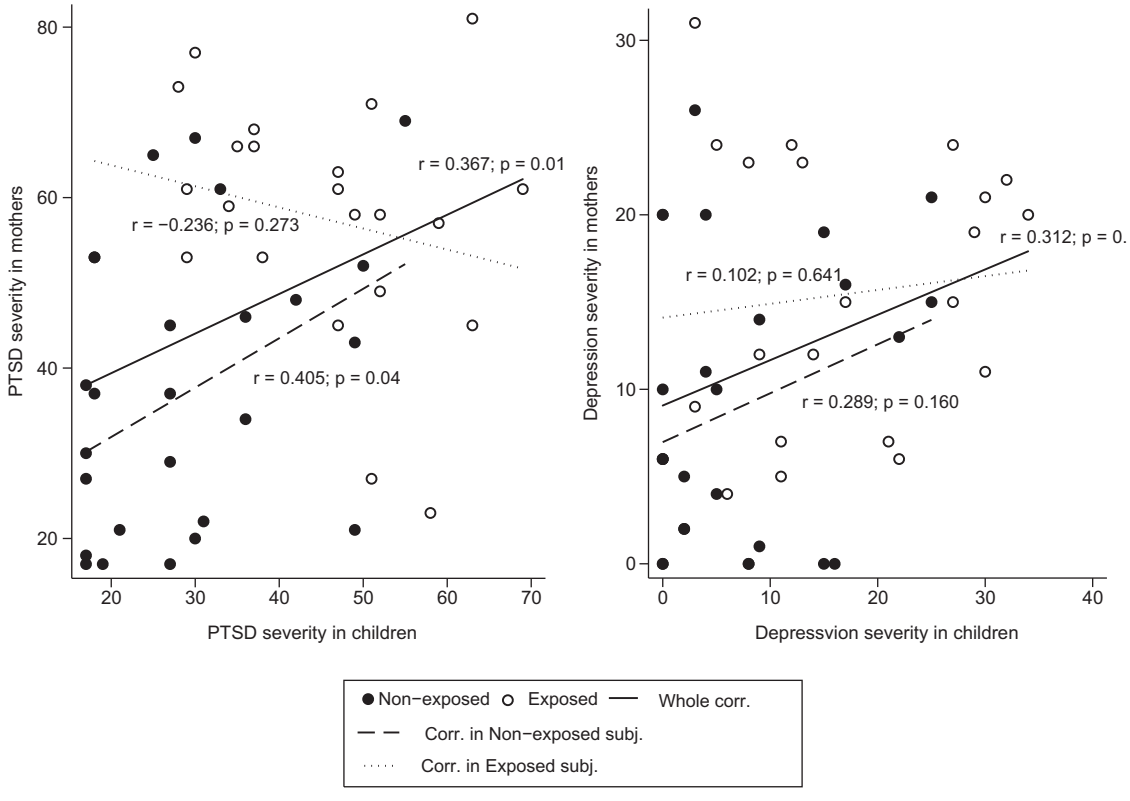

Figure 1. Correlation between PTSD severity in mothers and children (left) (Pearson $r=0.367$; $P=0.01$) and between depression severity as measured by the BDI in mothers and children (right) (Pearson's $r=0.312$; $P=0.03$).

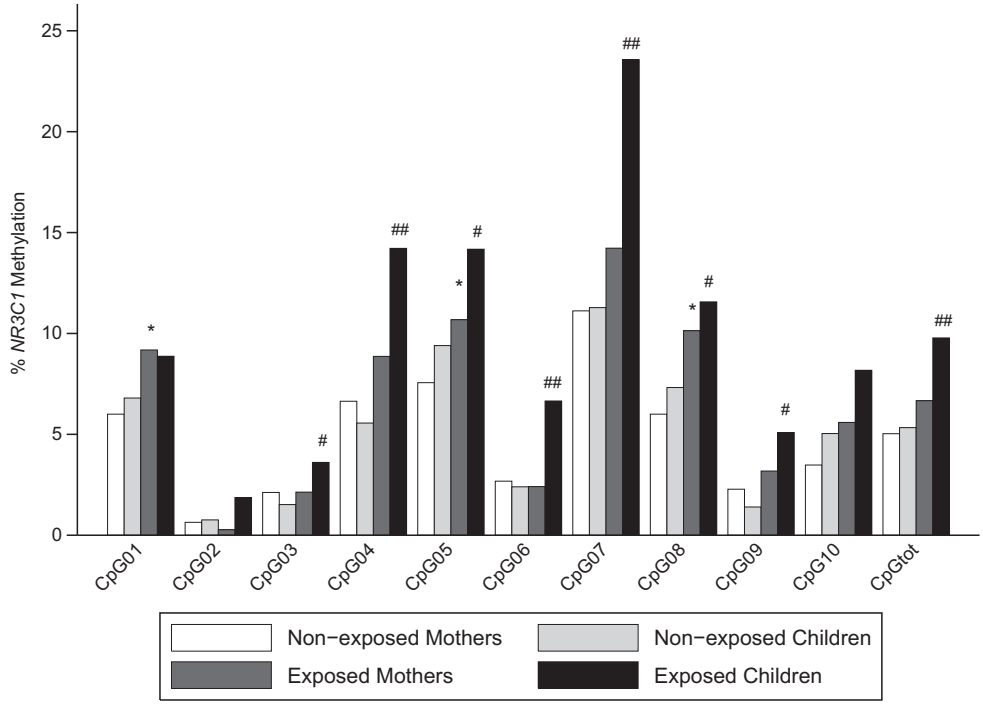

Figure 2. Percentage of *NR3C1* methylation for each CpG and mean of all CpGs (CpGtot) according to clinical status. $^{*}P<0.05$ for exposed mothers vs. non-exposed mothers; $^{\#}P<0.05$ for exposed children vs. non-exposed children; $^{\#\#}P<0.01$ for exposed children vs. non-exposed children.

(SD = 6.4) vs. 9.40 (SD = 7.6); $\beta = 0.71$; $P = 0.024$), CpG6 (6.65 (SD = 6.0) vs. 2.40 (SD = 3.0); $\beta = 0.91$; $P = 0.003$), CpG7 (23.56 (SD = 9.1) vs. 11.28 (SD = 8.9); $\beta = 1.24$; $P = 2.22*10^{-5}$), CpG8 (11.56 (SD = 7.5) vs. 7.32 (SD = 6.9); $\beta = 0.59$; $P = 0.047$), and CpG9 (5.09 (SD = 5.8) vs. 1.40 (SD = 3.9); $\beta = 0.76$; $P = 0.013$) (Figure 2).

There was a significant correlation between *NR3C1* methylation status in the mothers and methylation status in the children (Pearson's $r = 0.438$; $P = 0.002$) (Figure 3). Although the correlation was highly significant in non-exposed subjects (Pearson's $r = 0.872$; $P < 0.0001$), the correlation was not significant in the exposed group (Pearson's $r = -0.395$;

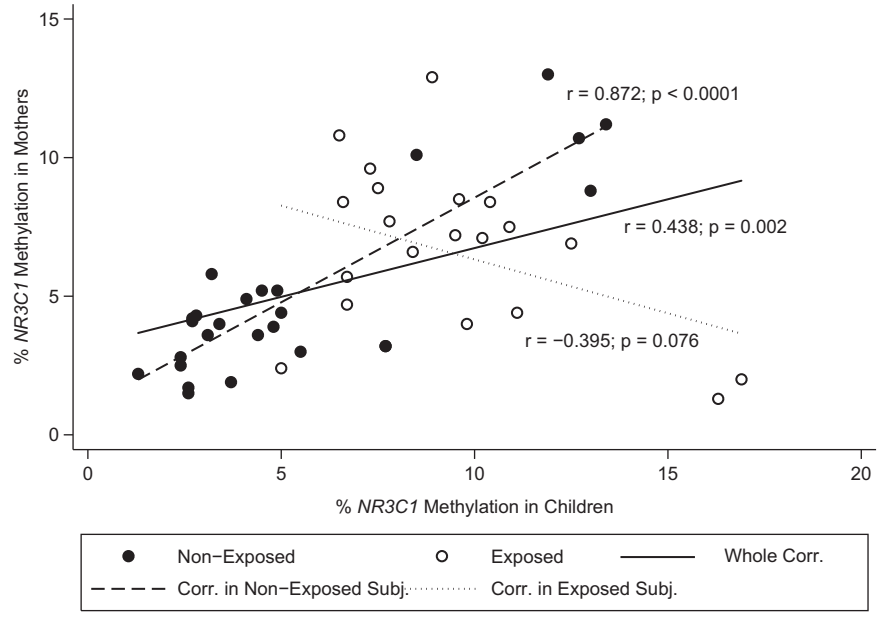

Figure 3. Correlation between *NR3C1* methylation status in mothers and in children (Pearson's $r = 0.438$; $P = 0.002$).

$P = 0.076$). This was better explained by a ceiling effect in the exposed group, the latter already having a high level of methylation.

In children, there was no sex-specific effect in DNA modification (7.61 (SD = 4.22) vs. 7.35 (SD = 4.23); $\beta = 0.07$; $P = 0.838$).

NR3C2 methylation status

Although not significant, mothers exposed to the genocide had lower methylation status (mean methylation of CpG1 to CpG8) of the *NR3C2* than non-exposed mothers (10.71 (SD = 2.9) vs. 10.95 (SD = 4.1); $\beta = -0.06$; $P = 0.843$). There was also no significant difference between exposed and non-exposed mothers for individual CpGs (considering CpG1 to CpG8). Children born from mothers exposed to the traumatic experience had lower methylation status of the *NR3C2* than children born from non-exposed mothers (7.76 (SD = 3.3) vs. 9.09 (SD = 3.2). This difference was nevertheless not significant ($\beta = 0.37$; $P = 0.237$). There was also no significant difference for any of the individual CpGs (Supplementary Figure 1 available at http://informahealthcare.com/doi/abs/10.3109/15622975.2013.866693). Interestingly, when looking at the CpGs for which fewer subjects were available and located within the body of the gene, we observed a increased methylation of *NR3C2* CpG15 to CpG24 in exposed mothers compared to non-exposed mothers (Supplementary Table I and Figure 2 available at http://informahealthcare.com/doi/abs/10.3109/15622975.2013.866693). No significant difference was observed between the two groups of children. There was no correlation between *NR3C2* methylation status in the mothers and methylation status in the children (Pearson's $r = 0.03$; $P = 0.876$).

In children, there was no sex-specific effect in DNA modification.

Cortisol levels

Exposed mothers as well as their children had lower cortisol levels than non-exposed mothers and their children, respectively (64.1 (SD = 22) vs. 101.4 (SD = 28), $t = 2.67$, $P = 0.006$ for comparison between mothers; 79.3 (SD = 21) vs. 106.5 (SD = 32), $t = 2.54$, $P = 0.003$ for comparison between children) (Figure 4). Not surprisingly, cortisol levels strongly correlated with PTSD and depression severity (Pearson's $r = 0.480$; $P = 0.002$ and Pearson's $r = 0.39$; $P = 0.004$).

Both *NR3C1* and *NR3C2* methylation status correlated with cortisol levels: the higher the methylation status, the lower the cortisol levels (Pearson's $r = -0.43$; $P = 0.003$ and Pearson's $r = -0.43$; $P = 0.01$).

Glucocorticoid receptor concentration

Exposed mothers as well as their children presented lower GR levels than non-exposed mothers and their children (177.41 (SD = 97.06) vs. 253.48 (SD = 83.92), $t = 2.77$; $P = 0.008$ for comparison between mothers; and 170.28 (SD = 57.18) vs. 246.00 (SD = 92.19); $t = 2.97$; $P = 0.006$ for comparison between children) (Figure 5). There was a

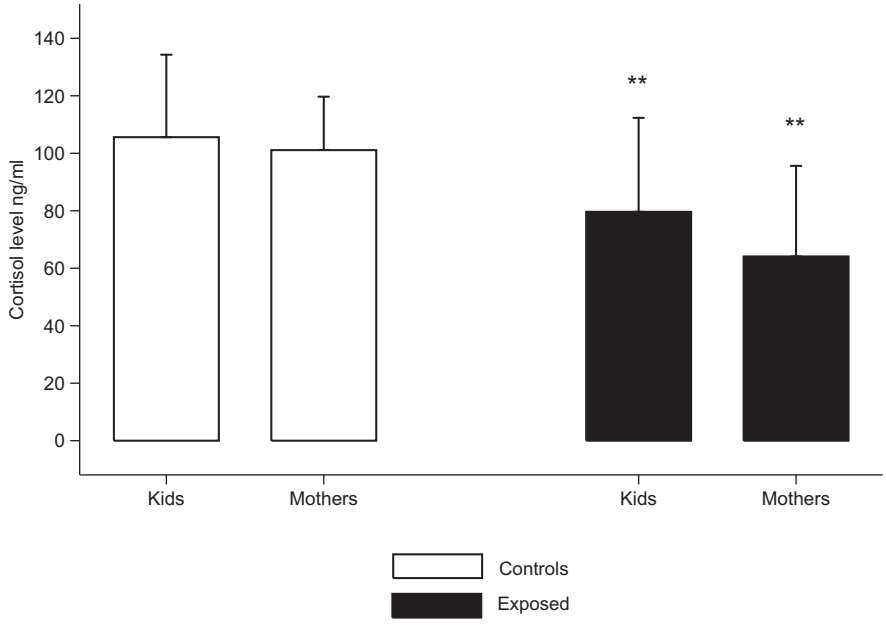

Figure 4. Plasma cortisol levels (ng/ml) in exposed vs. non-exposed mothers as well as their children. $^{**}P < 0.01$ for the comparison between exposed mothers and non-exposed mothers and for the comparison between exposed children and non-exposed children.

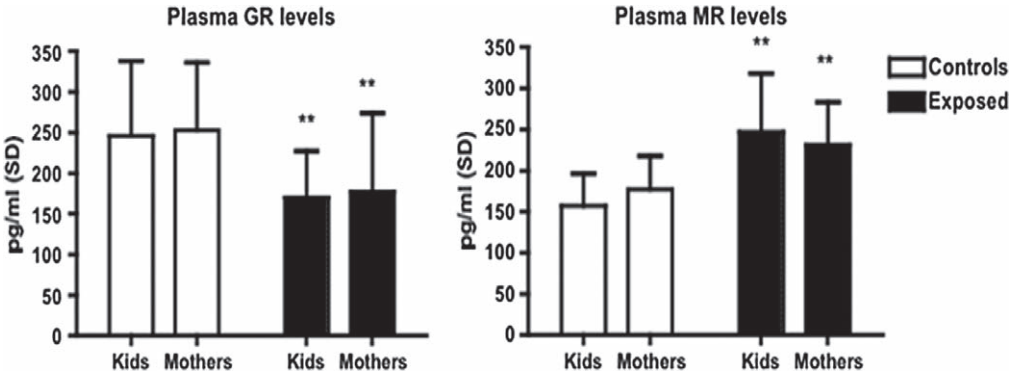

Figure 5. GR and MR levels in exposed vs. non-exposed mothers as well as their children. $^{**}P < 0.01$ for the comparison between exposed mothers and non-exposed mothers and for the comparison between exposed children and non-exposed children.

strong correlation between GR levels in mothers and GR levels in their children (Pearson's $r = 0.61$; $P = 0.007$).

Not surprisingly, there was a negative correlation between *NR3C1* methylation status and GR levels (Pearson's $r = -0.49$; $P = 0.003$).

Mineralocorticoid receptor concentration

Exposed mothers as well as their children had higher MR levels than non-exposed mothers and their children, respectively (231.23 (SD = 52.21) vs. 177.60 (SD = 41.91), $t = 4.00$; $P = 0.0002$ for comparison between mothers; and 247.47 (SD = 71.28) vs. 157.52 (SD = 39.02); $P < 0.0001$ for comparison between children) (Figure 5). There was a strong correlation between MR levels in mothers and MR levels in their children (Pearson's $r = 0.63$; $P < 0.003$) .

Surprisingly, MR levels did not correlate with *NR3C2* methylation status (Pearson's $r = 0.006$; $P = 0.972$) (considering CpGs 1–8).

No difference was found between males and females neither in GR levels nor in MR levels.

Discussion

In this study, we showed, in a sample of traumatized Tutsi widows and their children, that transgenerational impact of a trauma is a reality not only from a clinical point of view but also from a biological one.

We firstly replicated the results of several studies in the field showing that children born from mothers having been exposed to a traumatic experience and suffering from PTSD are at higher risk of negative mental health outcomes as adults (Malaspina et al. 2008; Brand et al. 2011). Interestingly and more closely related to our findings, Yehuda et al. and others have shown that maternal PTSD, in selected populations of refugees, war veterans, or victims of other major traumas, was associated with the development of PTSD in offspring (Yehuda and Bierer 2008; Roberts et al. 2012). Altogether, these findings add to the growing literature attributing sustained effect on mental health outcomes for offspring of maternal gestational stress.

We secondarily showed that transmission of PTSD to the offspring was associated with transmission of biological alterations of the HPA axis, specifically the blunted plasma cortisol levels. In line with these results, Yehuda et al. (2005) found that salivary cortisol levels were significantly lower in the offspring of women with PTSD directly exposed to the World Trade Center (WTC) collapse on 9/11 during their pregnancy than in those whose mothers had not developed PTSD. More generally, these results are consistent with several reports showing that PTSD and early-life adverse events are associated with specific profiles of HPA axis functioning such as high levels of corticotropin releasing factor (CRF), blunted urinary and plasma cortisol levels and increased cortisol suppression in response to dexamethasone administration (Elzinga et al. 2008; Carpenter et al. 2009; Yehuda and Bierer 2009; Tyrka et al. 2012). These data support the idea that prenatal stress has a profound effect on neurobehavioural development possibly through dysfunction of the HPA axis (Cottrell and Seckl 2009; Kinsella and Monk 2009).

Thirdly, we showed that PTSD in mothers was associated with epigenetic modifications of the *NR3C1* gene that were similarly found in their children. This higher methylation status of the *NR3C1* exon 1_F promoter region was associated with reduced GR levels in the blood. Early life adverse events have been shown to influence the methylation status of *NR3C1* in humans as well as in animals (Murgatroyd et al. 2009; Perroud et al. 2011). Although this is well established for events occurring early in life this has been investigated less for stress occurring during the gestational period. However, and consistent with our findings, these studies consistently found that

being exposed as a foetus to environmental stress is associated with a sustained increase in methylation status of the *NR3C1* promoter region (Oberlander et al. 2008; Radtke et al. 2011). For instance, Oberlander et al. (2008) found that prenatal exposure to increased third term maternal depressed/anxious mood was associated with increased methylation of *NR3C1*. The increased *NR3C1* methylation at this site was associated with increased salivary cortisol stress responses at 3 months. This suggests that exposure to maternal gestational stress may have a long lasting impact on the development of the child through increased GR promoter methylation which persists beyond infancy (Radtke et al. 2011). This may represent a foetal programming of the HPA axis which may be responsible for later psychological problems such as depression or PTSD as adults. Supporting this view, Mueller and Bale (2008) showed that, in mice, stress during pregnancy was associated in offspring with a depressive-like phenotype, and, in both the hypothalamus and amygdale, with changes in CRF and GR expression, and reduced methylation of the CRF gene. Their results further support the idea that mother's stress during pregnancy can influence, via epigenetic mechanisms, the foetal brain and behaviour development. Tobi et al. (2009) showed that prenatal famine was associated with changes in DNA methylation of several metabolic genes. Interestingly, they suggested that these changes depend on the gestational timing of the exposure. Several previous studies have suggested that the timing of the exposure during gestation is very important (Oberlander et al. 2008). Yehuda and co-workers (Yehuda et al. 2005; Yehuda and Bierer 2009) showed that the effect of PTSD on salivary cortisol levels in offspring was mainly observed for the second and third terms. In both animals and humans, exposure to glucocorticoids during the second and third terms of pregnancy, reduces offspring birth weight (Drake et al. 2005). This low birth weight following maternal stress has been associated with psychiatric problems (Susser et al. 1996). The mothers we studied were exposed to the traumatic event during the second and third terms of pregnancy and thus our results support the view of a timing effect of stress during pregnancy.

Our findings may at first glance appear striking, as with reduced availability of GR one would expect to find higher levels of cortisol due to reduced negative feedback. This relationship may not be so straightforward since firstly corticoids regulate GR and are further regulated by them; and secondly because glucocorticoids also exert their actions through MR and not only through GR. Our data support other studies in the field showing that MR plays an important role in the pathology of PTSD. For instance, Zhang et al. (2012) found that rats exposed to a single prolonged stress, an established model for PTSD, showed an increased expression of MR in their medial prefrontal cortex. These results were confirmed by other studies (Gesing et al. 2001; Li et al. 2011). MR has a high affinity for cortisol and are sufficient to maintain low basal corticosterone levels during the circadian. GR has less affinity and is occupied during increased levels of glucocorticoids or during acute stress (de Kloet et al. 2005). Via these GR, glucocorticoids inhibit the neuroendocrine stress response via GR in the pituitary, hypothalamus and hippocampus. However, more and more data support the non-negligible role of MR in mediating the stress response in humans indicating that MR contribute to the regulation of the HPA axis during stress possibly by potentiating the effects of glucocorticoids on GR (Spencer et al. 1998). These studies suggest that a GR:MR imbalance, primarily resulting from the reduced GR availability due to increased *NR3C1* methylation, may increase susceptibility to stress-related psychiatric disorders such as PTSD (Harris et al. 2012; Mulligan et al. 2012). MR may, in that sense, be increased as a compensatory mechanism, though not entirely efficient, to counterbalance the reduced numbers of GR and correct the GR:MR balance. Thus, animal as well as clinical observations point towards an involvement of both GR and MR in PTSD and support the idea that activation of both MR and GR contribute to the inhibitory effect of glucocorticoids that attenuate the magnitude and duration of stress-induced HPA axis activity (Kellner et al. 2002). Some authors (Yehuda and Bierer 2009) have suggested that PTSD is associated with greater number of GR possibly due to hypomethylation of the *NR3C1* as an explanation for the low cortisol levels in PTSD. We convincingly show here that PTSD is associated with increased methylation of *NR3C1* and higher GR levels. What may differ between PTSD and other studies on stress showing higher reactivity of the HPA axis and lower availability of GR (Oberlander et al. 2008; McGowan et al. 2009) is the possible role played by the MR. Nevertheless, this explanation is far from being convincing as our results do not entirely support it. Although, we indeed found higher MR levels as well as lower, but not significant, *NR3C2* methylation status in exposed subjects compared to non-exposed ones, the correlation between these two measures was not significant (at least for CpGs 1–8 located in the promoter region of the gene). Moreover, we were not able to show, contrary to *NR3C1*, a transgenarational transmission of epigenetic modifications of *NR3C2*. These findings thus suggest a more complex relationship that still needs to be unrevealed but in which both *NR3C1* and *NR3C2* and their

corresponding products still play a central role. Interestingly, for some CpGs located within the body of the gene, we found higher methylation in exposed mothers than in non-exposed mothers. This result might in part explain the higher MR levels found in exposed mothers as higher methylation of a gene body has been shown to activate expression of that gene (Jones 2012). However due to technical difficulties to correctly assess these CpGs in our sample and thus to the limited sample size for these sites, these results should be considered with caution.

Another explanation that may help understand the complex relation between GR promoter modification, gene expression and HPA stress responsivity is the role of other DNA modifications such as phosphorylation, hydroxymethylation of cytosine that is not distinguished from the methylation by the method used in this paper and/or demethylation (5-carboxylcytosine and 5-formylcytosine). Future research should try to better define all the DNA modification that may be involved in the regulation of such a complex system.

An additional point that might help explaining our results is that, although we measured the active form of cortisol, we cannot rule out an effect of other forms of cortisol (i.e., bound to other proteins such as corticosteroid binding globulin). These other forms might have biased the level of cortisol and readers should keep this in mind when interpreting our findings.

This study has several limitations, one being the retrospective assessment of PTSD and a second being the absence for instance of *NR3C2* methylation status or thorough investigation, at the epigenetic and at the biological level, of all the proteins involved in the regulation of the HPA axis. A third one is the investigation in periphery of what is happening in the brain. From these perspectives we are having a proxy of a more complex story on the relationship between HPA axis and PTSD. But given the fairly comprehensive results of our study and the relatively unique sample studied, we are quite confident in these preliminary findings that definitely need to be extended to other samples with similar traumatic events. A fourth limitation lies in the lack of other environmental measures that might have influenced our findings such as medical health of the participants, past and current medication, substance use, and socio-economic status of the non-exposed group for instance. This study was designed to measure the impact of a strong environmental factor, exposure to genocide, and not those of less striking environmental events. We thus believe that, whatever was the other underlying environment, the difference we observed is due to exposure or not to the genocide, an environmental exposure that overwhelm the others in term of epigenetic impact. Fifthly, data were collected retrospectively and in a cross-sectional manner, and thus our findings should be taken with this caveat in mind. Future researches in the field should try to collect prospective data of exposed and non-exposed individuals over the years in order to definitely link the epigenetic and biological modifications to exposure to trauma. Finally, the PCL-17 is used to assess severity of current PTSD but not the presence or absence of a diagnostic of PTSD. Our results should thus be understood as a transmission of symptoms of PTSD rather than the disorder itself.

Although we showed the transmission of PTSD and of altered HPA axis functioning, it remains difficult to answer what is really transmitted by the mother. Is it firstly the environment that the mother with PTSD is creating for her child, her way of living, of dealing with the stress, being too protective of her child and not letting him/her naturally undergo voluntary separation resulting in less secure attachments and poor emotion regulation (Yehuda and Bierer 2008; Yehuda and Bierer 2009; Brand et al. 2011), being hyper-vigilant and "transmitting" this tendency to the child who will secondarily develop PTSD and HPA axis alterations? As suggested by Yehuda et al. (2009) this may be seen as an adaptive strategy to offspring who continue to live in a dangerous environment as equally unfavourable as that of their parents. Or does maternal PTSD confer a risk of PTSD on the offspring firstly through epigenetic modifications and biological alteration of the HPA axis and only secondarily leads to the development of PTSD? Possibly the two phenomena play and interact together in order to unfortunately ensure the transmission of PTSD. We thus may postulate that PTSD negatively affects parenting and epigenetic programming such that children of parents with PTSD are more likely to suffer from PTSD (Ozer et al. 2003; Roberts et al. 2012).

In conclusion, our study shows that abnormal HPA axis activity may play a central role in the pathophysiology of PTSD and depression and that the mechanisms underlying these dysfunctions, including epigenetic modifications, would be sufficient to explain the transgenerational transmission of this disorder from the mother to the offspring. Our study also highlights the enduring impact of the Tutsi genocide almost 20 years after this dramatic event and shows that PTSD and depression remain a significant public health outcome. A better understanding, at the biological and clinical levels, of the mechanisms involved in PTSD and in its transmission across generations should help prevent the development of this disorder in subjects exposed to traumatic events.

Acknowledgements

We thank the participants and the Tutsi genocide widows' associations for their participation. The Lab analyses were performed by Rafael Fernandez and Eladia Ballmann (Geneva) and Seraphine Murorunkwere (Rwanda). We would like to thank Christelle Stouder for her help in the analyses.

Statement of Interest

NP and AM have received speaker's honoraria from Lundbeck. There is no conflict of interest with the content of this manuscript. This research was supported by the AXA Research Foundation "Vulnerability to risky behaviors as anomalies in neuro-developmental trajectories: an individualized approach" to AM.

References

Brand SR, Engel SM, Canfield RL, Yehuda R. 2006. The effect of maternal PTSD following in utero trauma exposure on behavior and temperament in the 9-month-old infant. Ann Y Acad Sci 1071:454–458.

Brand SR, Schechter JC, Hammen CL, Brocque RL, Brennan, PA. 2011. Do adolescent offspring of women with PTSD experience higher levels of chronic and episodic stress? J Trauma Stress 24:399–404.

Bromer C, Marsit CJ, Armstrong DA, Padbury JF, Lester B. 2013. Genetic and epigenetic variation of the glucocorticoid receptor (NR3C1) in placenta and infant neurobehavior. Dev Psychobiol 55:673–683.

Carpenter LL, Tyrka AR, Ross NS, Khoury L, Anderson GM, Price LH. 2009. Effect of childhood emotional abuse and age on cortisol responsivity in adulthood. Biol Psychiatry 66:69–75.

Collet L, Cottraux J. 1986. [The shortened Beck depression inventory (13 items). Study of the concurrent validity with the Hamilton scale and Widlocher's retardation scale]. Encephale 12:77–79.

Cottrell EC, Seckl JR. 2009. Prenatal stress, glucocorticoids and the programming of adult disease. Front Behav Neurosci 3:19.

de Kloet ER, Joels M, Holsboer F. 2005. Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463–475.

Drake AJ, Walker BR, Seckl JR. 2005. Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. Am J Physiol Regul Integr Comp Physiol 288:R34–38.

Elzinga BM, Roelofs K, Tollenaar MS, Bakvis P, van Pelt J, Spinhoven P. 2008. Diminished cortisol responses to psychosocial stress associated with lifetime adverse events a study among healthy young subjects. Psychoneuroendocrinology 33:227–237.

Filiberto AC, Maccani MA, Koestler D, Wilhelm-Benartzi C, Avissar-Whiting M, Banister CE, et al. 2012. Birthweight is associated with DNA promoter methylation of the glucocorticoid receptor in human placenta. Epigenetics 6:566–572.

Gesing A, Bilang-Bleuel A, Droste SK, Linthorst AC, Holsboer F, Reul JM. 2001. Psychological stress increases hippocampal mineralocorticoid receptor levels: involvement of corticotropin-releasing hormone. J Neurosci 21:4822–4829.

Harris AP, Holmes MC, de Kloet ER, Chapman KE, Seckl JR. 2013. Mineralocorticoid and glucocorticoid receptor balance in control of HPA axis and behaviour. Psychoneuroendocrinology 38:648–658.

Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 13: 484–492.

Kellner M, Baker DG,Yassouridis A, Bettinger S, Otte C, Naber D, et al. 2002. Mineralocorticoid receptor function in patients with posttraumatic stress disorder. Am J Psychiatry 159:1938–1940.

Kinsella MT, Monk C. 2009. Impact of maternal stress, depression and anxiety on fetal neurobehavioral development. Clin Obstet Gynecol 52:425–440.

Li M, Han F, Shi Y. 2011. Expression of locus coeruleus mineralocorticoid receptor and glucocorticoid receptor in rats under single-prolonged stress. Neurol Sci 32:625–631.

Malaspina D, Corcoran C, Kleinhaus KR, Perrin MC, Fennig S, Nahon D, et al. 2008. Acute maternal stress in pregnancy and schizophrenia in offspring: a cohort prospective study. BMC Psychiatry 8:71.

McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, et al. 2009. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci 12:342–348.

Mueller BR, Bale TL. 2008. Sex-specific programming of offspring emotionality after stress early in pregnancy. J Neurosci 28:9055–9065.

Mulligan CJ, D'Errico NC, Stees J, Hughes DA. 2012. Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. Epigenetics 7:853–857.

Munyandamutsa N, Mahoro Nkubamugisha P, Gex-Fabry M, Eytan A. 2012. Mental and physical health in Rwanda 14 years after the genocide. Soc Psychiatry Psychiatr Epidemiol 47: 1753–1761.

Murgatroyd C, Patchev AV, Wu Y, Micale V, Bockmuhl Y, Fischer D, et al. 2009. Dynamic DNA methylation programs persistent adverse effects of early-life stress. Nat Neurosci 12:1559–1566.

Neugebauer R, Fisher PW, Turner JB, Yamabe S, Sarsfield JA, Stehling-Ariza T. 2009. Post-traumatic stress reactions among Rwandan children and adolescents in the early aftermath of genocide. Int J Epidemiol 38:1033–1045.

Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. 2008. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 3:97–106.

Ozer EJ, Best SR, Lipsey TL, Weiss DS. 2003. Predictors of posttraumatic stress disorder and symptoms in adults: a meta-analysis. Psychol Bull 129:52–73.

Perroud N, Paoloni-Giacobino A, Prada P, Olie E, Salzmann A, Nicastro R, et al. 2011. Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: a link with the severity and type of trauma. Transl Psychiatry 1:e59.

Radtke K, Ruf M, Gunter H, Dohrmann K, Schauer M, Meyer A, et al. 2011. Transgenerational impact of intimate partner violence on methylation in the promoter of the glucocorticoid receptor. Transl Psychiatry 1:e21. doi: 10.1038/tp.2011.21.

Roberts AL, Galea S, Austin SB, Cerda M, Wright RJ, Rich-Edwards JW, et al. 2012. Posttraumatic stress disorder across two generations: concordance and mechanisms in a population-based sample. Biol Psychiatry 72:505–511.

Ruggiero KJ, Rheingold AA, Resnick HS, Kilpatrick DG, Galea S. 2006. Comparison of two widely used PTSD-screening

instruments: implications for public mental health planning. J Trauma Stress 19:699–707.

Schaal S, Dusingizemungu JP, Jacob N, Elbert T. 2011. Rates of trauma spectrum disorders and risks of posttraumatic stress disorder in a sample of orphaned and widowed genocide survivors. Eur J Psychotraumatol 2. doi: 10.3402/ejpt.v2i0.6343.

Spencer RL, Kim PJ, Kalman BA, Cole MA. 1998. Evidence for mineralocorticoid receptor facilitation of glucocorticoid receptor-dependent regulation of hypothalamic-pituitary-adrenal axis activity. Endocrinology 139:2718–2726.

Susser E, Neugebauer R, Hoek HW, Brown AS, Lin S, Labovitz D, et al. 1996. Schizophrenia after prenatal famine. Further evidence. Arch Gen Psychiatry 53:25–31.

Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, et al. 2009. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet 18:4046–4053.

Tyrka AR, Price LH, Marsit C, Walters OC, Carpenter LL. 2012. Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. PLoS One 7:e30148.

Yehuda R, Bierer LM. 2008. Transgenerational transmission of cortisol and PTSD risk. Prog Brain Res 167:121–135.

Yehuda R, Bierer LM. 2009. The relevance of epigenetics to PTSD: implications for the DSM-V. J Trauma Stress 22:427–434.

Yehuda R, Engel SM, Brand SR, Seckl J, Marcus SM, Berkowitz GS. 2005. Transgenerational effects of posttraumatic stress disorder in babies of mothers exposed to the World Trade Center attacks during pregnancy. J Clin Endocrinol Metab 90:4115–4118.

Yehuda R, Teicher MH, Seckl JR, Grossman RA, Morris A, Bierer LM. 2007. Parental posttraumatic stress disorder as a vulnerability factor for low cortisol trait in offspring of holocaust survivors. Arch Gen Psychiatry 64:1040–1048.

Zhang JH, Han F, Shi YX. 2012. Single prolonged stress induces changes in the expression of mineralocorticoid receptor in the medial prefrontal cortex in a rat model of post-traumatic stress disorder. Mol Med Report 6:330–334.

Supplementary data available online

Supplementary Figures and Table